

# Calcium-Calmodulin-dependent Protein Kinase II and Protein Kinase C-mediated Phosphorylation and Activation of D-myo-Inositol 1,4,5-Trisphosphate 3-Kinase B in Astrocytes\*

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D-myo-Inositol 1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>) 3-kinase catalyzes the production of D-myo-inositol 1,3,4,5-tetrakisphosphate from the second messenger Ins(1,4,5)P<sub>3</sub>. Transient and okadaic acid-sensitive activation of Ins(1,4,5)P<sub>3</sub> 3-kinase by 8–10-fold is observed in homogenates prepared from rat cortical astrocytes after incubation with either carbachol or UTP. 12-O-Tetradecanoylphorbol-13-acetate provokes the activation of Ins(1,4,5)P<sub>3</sub> 3-kinase by 2-fold in both cell systems. The kinase was purified by calmodulin-Sepharose from the two cell systems. Enzyme activity corresponding to the silver-stained 88-kDa protein could be regenerated after SDS-polyacrylamide gel electrophoresis. Antibodies to two distinct peptides chosen in the primary structure of human Ins(1,4,5)P<sub>3</sub> 3-kinase B recognized the astrocytic native isoform. In [<sup>32</sup>P]orthophosphate-preincubated cells, a major phosphorylated 88-kDa enzyme could be purified and identified in cells in response to receptor activation or 12-O-tetradecanoylphorbol-13-acetate treatment. Calmodulin kinase II inhibitors (*i.e.* KN-93 and KN-62) and a protein kinase C inhibitor (*i.e.* calphostin C) prevented the phosphorylation of the 88-kDa isoenzyme. In addition to enzyme activation, a redistribution of Ins(1,4,5)P<sub>3</sub> 3-kinase from soluble to particulate fraction of astrocytes was observed. *In vitro* phosphorylation of the purified enzyme by calmodulin kinase II and protein kinase C added together resulted in a maximal 60–70-fold activation.

Hydrolysis of the plasma membrane phosphatidylinositol 4,5-bisphosphate and the formation of D-myo-inositol 1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>)<sup>1</sup> result from the activation of a wide variety of cell surface receptors by neurotransmitters and hormones (1). Ins(1,4,5)P<sub>3</sub> 3-kinase occupies a crucial position

in this signal transduction cascade, inasmuch as it catalyzes a rapid production of D-myo-inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P<sub>4</sub>) and generates a metabolic pathway for many highly phosphorylated inositol phosphates. D-myo-inositol 3,4,5,6-tetrakisphosphate displays a second messenger function by selectively blocking epithelial Ca<sup>2+</sup>-activated chloride channels (2–4). The production of Ins(1,3,4,5)P<sub>4</sub> in response to receptor activation has been observed in various cell types, *i.e.* in rat brain cortical slices (5, 6), in human astrocytoma 1321N1 cell line (7), and in primary cultures of rat astrocytes (8). Due in large part to the efforts of Irvine and colleagues, there is experimental evidence for a possible second messenger function of Ins(1,3,4,5)P<sub>4</sub> in regulating Ca<sup>2+</sup> homeostasis at least in certain cell types (9, 10). Recently, Ca<sup>2+</sup>-dependent K<sup>+</sup> and Cl<sup>−</sup> channels have been shown to be activated by the non-metabolizable D-myo-inositol 2,4,5-trisphosphate and Ins(1,3,4,5)P<sub>4</sub> in primary cultures of mouse lacrimal acinar cells (11). Its high affinity and high isomeric specificity of binding to a GTPase-activating protein (GAP) (Ras-GAP1<sup>IP4BP</sup> isolated from pig platelets) have been reported (12). An increase in D-myo-inositol 2,4,5-trisphosphate-mediated Ca<sup>2+</sup> mobilization in the presence of Ins(1,3,4,5)P<sub>4</sub> has been reported in permeabilized L-1210 cells. This effect probably involves the participation of GAP1<sup>IP4BP</sup> acting together with an activated monomeric G-protein (13).

Molecular heterogeneity has been demonstrated for Ins(1,4,5)P<sub>3</sub> 3-kinase; cloning of cDNAs encoding rat and human Ins(1,4,5)P<sub>3</sub> 3-kinase A (14–16) as well as rat and human Ins(1,4,5)P<sub>3</sub> 3-kinase B (17–19) has been reported. In brain, isoform A is highly expressed in neuronal cells of the cortex, hippocampus, and cerebellum in both rat and human (20, 21). Isoform A is mainly expressed in the dendrites of hippocampal CA1 cells, and several reports suggested a second messenger role for Ins(1,3,4,5)P<sub>4</sub> in neuronal cells, *e.g.* Ins(1,3,4,5)P<sub>4</sub>-mediated Ca<sup>2+</sup> entry in CA1 cells, with the suggestion it may contribute to ischemia-induced neuronal death (22). The distribution of mRNA corresponding to rat Ins(1,4,5)P<sub>3</sub> 3-kinase B has been shown to be more general as compared with isoform A (19). In human brain, *in situ* hybridization data indicated that mRNA corresponding to isoenzyme B was mostly present in astrocytic cells, in contrast to the neuronal isoenzyme A (21). However, this last point has never been established at the protein level.

Ins(1,4,5)P<sub>3</sub> 3-kinase purified from a large number of cell types appeared to be sensitive to the Ca<sup>2+</sup>-CaM complex, *i.e.* 2-fold in rat and human brain (isoform A) (15, 16, 23) to 17-fold in human platelets (24). The primary structures of rat and human Ins(1,4,5)P<sub>3</sub> 3-kinases A and B reveal the presence of various potential phosphorylation sites based on consensus phosphorylation site sequences for Ca<sup>2+</sup>-CaM-dependent protein kinase II (CaM kinase II), protein kinase C (PKC), and

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<sup>1</sup> The abbreviations used are: Ins(1,4,5)P<sub>3</sub>, D-myo-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P<sub>4</sub>, D-myo-inositol 1,3,4,5-tetrakisphosphate; GAP, GTPase-activating protein; PKC, protein kinase C; PKA, protein kinase A; CaM, calmodulin; CaM kinase, Ca<sup>2+</sup>-CaM-dependent protein kinase II; PAGE, polyacrylamide gel electrophoresis; TPA, 12-O-tetradecanoylphorbol-13-acetate.

cAMP-dependent protein kinase (PKA) (15–17). *In vitro* experiments demonstrated that Ins (1,4,5)P<sub>3</sub> 3-kinases A (25) and B (26) are substrates for PKC- and PKA-mediated phosphorylation. Ins (1,4,5)P<sub>3</sub> 3-kinase A is the target of a regulatory mechanism involving CaM kinase II-mediated phosphorylation both *in vitro* and in intact cells (27). Treatment of rat brain cortical slices with carbachol provoked an increase in the phosphorylation of Ins (1,4,5)P<sub>3</sub> 3-kinase A and a corresponding increase in enzymic activity (3–5-fold) and in CaM affinity (25-fold). An activation of Ins (1,4,5)P<sub>3</sub> 3-kinase has also been observed in rat brainstem slices in response to serotonin through the CaM kinase II pathway. This effect was also sensitive to okadaic acid (28).

The physiological relevance of the existence of multiple isoforms of Ins (1,4,5)P<sub>3</sub> 3-kinase has not yet been pointed out experimentally in intact cells, especially concerning specific cellular and subcellular protein distributions and concerning distinct regulatory mechanisms with variable functional consequences. Data in the present report revealed for the first time at the protein level that the astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase corresponds to isoform B. We have shown that the activation mechanism of Ins (1,4,5)P<sub>3</sub> 3-kinase A can be generalized to Ins (1,4,5)P<sub>3</sub> 3-kinase B in astrocytes. It nevertheless involves other mediators with distinct functional consequences. Activation of this isoform was provoked by PKC and CaM kinase II-mediated phosphorylation *in vitro* and in intact cells. In this study, direct evidence is provided for a novel regulatory mechanism involving phosphorylation by both PKC and CaM kinase II, activation and redistribution of Ins (1,4,5)P<sub>3</sub> 3-kinase B in intact astrocytes.

#### EXPERIMENTAL PROCEDURES

**Generation of Antibodies Raised against the C-terminal End of Human Ins (1,4,5)P<sub>3</sub> 3-Kinase Isoform B**—An immune serum recognizing a 20-amino acid peptide (*i.e.* SGLNNLVLDILTEMSQDAPLA) corresponding to the last 20 amino acids of human isoenzyme B has been generated. The peptide has been coupled to hemocyanin in the presence of glutaraldehyde and injected to rabbits. The antibodies (dilution 1:1000) immunodetected recombinant isoform B but not isoform A after Western blotting.<sup>2</sup> Another antiserum (dilution 1:500) raised against a 15-amino acid peptide (*i.e.* CPPGDRVGVQPGNSR) in the N-terminal region of human isoform B has been described before (24). For competition studies, 5 µg of the corresponding peptides were added for 1 h to the diluted serum before immunodetection.

**Preparation of Rat Brain Cortical Astrocytes**—Primary cultures of rat cerebral cortex astrocytes were established using dissociated rat cerebral tissue at 2 or 3 days after birth, according to methods previously described (29). Briefly, dissected cortical tissue was washed and then dissociated by gentle repeated pipetting in modified Eagle's medium containing 1 mM sodium pyruvate, 10% fetal calf serum, 2% penicillin/streptomycin, and 1% fungizone. Cells were decanted by gravity for 5 min, and the supernatant was saved. The pellet was dissociated again, and the supernatant was added to the first one. Cells were diluted in complemented cell medium and plated in 9-cm diameter or 24 × 24-cm<sup>2</sup> dishes at 37 °C with 5% CO<sub>2</sub>. When astrocytes were adherent, dishes were vigorously agitated overnight and the medium was changed after two washes. Astrocytes reached confluence after 7 days in culture and could be trypsinized three to four times. Human astrocytoma 1321N1 cells were grown in the same complemented medium. Cell culture medium, dishes, and antibiotics were from Life Technologies, Inc.

**Incubations of Cortical Astrocytes and 1321N1 Cells**—When the astrocytic cells were ~80% subconfluent (7–8 × 10<sup>6</sup> cells in 8 ml of culture medium), they were washed twice with 2 ml of prewarmed KRH medium (124 mM NaCl, 5 mM KCl, 1.25 mM MgSO<sub>4</sub>, 1.45 mM CaCl<sub>2</sub>, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM glucose, 25 mM Hepes/NaOH, pH 7.4). A 2-ml aliquot of the same prewarmed medium containing the agent(s) was pipetted onto each culture dish. Cell incubations were terminated by aspirating the incubation medium prior to rinsing rapidly the cells twice with KRH medium. Cells were harvested by scraping with a rubber policeman in

200 µl of ice-cold lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM KCl, 12 mM 2-mercaptoethanol, 0.5% Nonidet P-40, 100 mM NaF, 50 mM okadaic acid, 1 mM sodium orthovanadate, 0.1 mM Pefabloc, 5 µM leupeptin, and 15 µg/ml calpain inhibitors I and II). Final cell lysates were obtained by three successive cycles of freeze/thaw. Ins (1,4,5)P<sub>3</sub> 3-kinase activity was measured at 5 µM Ins (1,4,5)P<sub>3</sub> (30). *K<sub>m</sub>* values for Ins (1,4,5)P<sub>3</sub> were estimated by measuring initial velocities in the presence of 0–50 µM Ins (1,4,5)P<sub>3</sub> and by using a nonlinear least-squares curve fitting of substrate-velocity relationships (Marquardt-Levenberg algorithm). Sensitivity of the enzyme for CaM was measured by assaying enzymic activity at 5 µM Ins (1,4,5)P<sub>3</sub>, 10 µM free Ca<sup>2+</sup>, and increasing concentrations of CaM (0–2 µM). Okadaic acid, sodium orthovanadate, NaF, ATP, UTP, carbachol, histamine, tetradecanoyl phorbol acetate (TPA), Nonidet P-40, and leupeptin were from Sigma. Pefabloc was from Pentapharm. Calpain inhibitors I and II were from Roche Molecular Biochemicals. KN-93, KN-62, calphostin C, and forskolin were from Calbiochem. [<sup>3</sup>H]Ins (1,4,5)P<sub>3</sub> (15–30 Ci/mmol) was from NEN Life Science Products. SDS-PAGE, Western blotting and immunodetection were performed as described previously (23).

**Cortical Astrocyte or 1321N1 Cell Labeling and Enzyme Purification by CaM-Sepharose**—When cells were ~80% subconfluent in 6-cm diameter culture dishes, they were washed two times and incubated for 2 h in Dulbecco's modified Eagle's pyrophosphate-free medium supplemented with carrier-free [<sup>32</sup>P]orthophosphate (0.5 mCi/ml). The cells were subsequently washed in prewarmed KRH medium, and a 2-ml aliquot of this medium containing the agent(s) was pipetted onto each culture dish for an incubation with agonist. Cell extracts were prepared as described above. Astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase was isolated after each incubation by CaM-Sepharose (Amersham Pharmacia Biotech) (27). The 200-µl cell extract (~1.9 mg of total protein) was applied in the presence of 0.5 mM CaCl<sub>2</sub> to a column containing 200 µl of CaM-Sepharose eluted by gravity at 4 °C. The column was washed with 5 ml of equilibration buffer (50 mM Tris/HCl pH 7.4, 12 mM 2-mercaptoethanol, 0.1 M NaCl, 0.2 mM CaCl<sub>2</sub>, 0.1% Triton X-100, 100 mM NaF, 50 mM okadaic acid, 1 mM sodium vanadate, 0.1 mM Pefabloc, 5 µM leupeptin, and 15 µg/ml calpain inhibitors I and II) containing no Triton X-100 (Roche Molecular Biochemicals). The column was washed in the same buffer with 0.5 mM EGTA and no Triton X-100. Specific elution of Ins (1,4,5)P<sub>3</sub> 3-kinase was performed in the presence of 2 mM EGTA, 1% Triton X-100, as well as phosphatase and protease inhibitors as described before (24, 27). The two most active fractions (total volume of 400 µl) were concentrated to 50 µl using Strataclean resin (Stratagene), and proteins were separated by SDS-PAGE and detected by autoradiography using a Hyperfilm-MP (Amersham Pharmacia Biotech) exposed for 24 h.

For immunodetection and *in vitro* phosphorylation experiments, primary cultures of cortical astrocytes and 1321N1 cell line were cultured in monolayers in square cell culture dishes (24 cm × 24 cm). The cell crude extracts were applied in the presence of 1 mM CaCl<sub>2</sub> onto 40 ml of CaM-Sepharose. Purified astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase was eluted in the presence of 2 mM EGTA, 1% Triton X-100, together with phosphatase and protease inhibitors. Fractions presenting the highest Ins (1,4,5)P<sub>3</sub> 3-kinase activity (3 fractions, 25 ml) were concentrated by Amicon Centricon-10 to obtain a final 50-µl sample (~3 µg of purified enzyme, with a specific activity of 1.4 ± 0.2 µmol/min × mg as determined at 5 µM Ins (1,4,5)P<sub>3</sub> in the absence of the Ca<sup>2+</sup>-CaM complex).

**Separation of Soluble and Particulate Fractions in Astrocytic Cells**—Homogenates prepared from rat cortical astrocytes or 1321N1 cells (7–8 × 10<sup>6</sup> cells in 8 ml of culture medium per incubation condition) were prepared by three successive cycles of freeze/thaw in the lysis buffer without detergent. After 10 passages in a 26-gauge needle attached to a 1-ml syringe, cell homogenates were centrifuged at 4 °C for 1 h at 100,000 × *g*. The supernatant (cytosol fraction) was saved; the pellet (particulate fraction; ~30 µl) was washed three times and recovered in an equal final volume of the same lysis buffer before assay of enzymic activity. For Western blot studies, the same procedure was followed to separate the soluble and particulate fractions from homogenates prepared from 2.3 × 10<sup>8</sup> 1321N1 cells (per incubation condition). Ins (1,4,5)P<sub>3</sub> 3-kinase activity was solubilized from the particulate fraction under strong agitation in the lysis buffer (1 ml) containing 1% Triton X-100 for 1 h at 4 °C. The solubilized fraction was recovered after centrifugation at 100,000 × *g* for 1 h at 4 °C. Enzyme activity assay showed that solubilization of Ins (1,4,5)P<sub>3</sub> 3-kinase from the particulate fraction was efficient at approximately 80–85% as compared with the total particulate activity (Fig. 7A). The soluble (12 mg of protein) and the solubilized particulate (75 mg of protein) fractions (1 ml each) were adjusted to 0.1% Triton X-100 and 0.5 mM CaCl<sub>2</sub> in a final volume of 50 ml and purified separately by CaM-Sepharose as described above. The

<sup>2</sup> V. Dewaste, unpublished data.

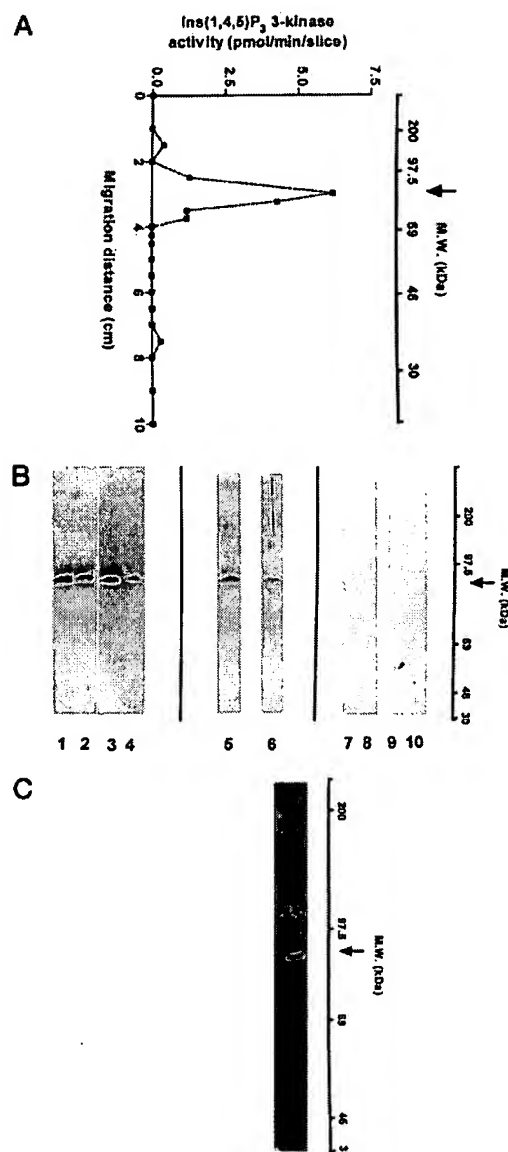
active fractions were concentrated by Amicon Ultrafree-15 and applied for SDS-PAGE. Western blotting and immunodetections were performed as described above.

**In Vitro Enzyme Phosphorylation by CaM Kinase II and PKC**—Phosphorylation of purified astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase (100 ng) by rat brain CaM kinase II (2 ng; Calbiochem) and/or rat brain PKC (2 ng) was performed as described before (27). Phosphorylation samples were stopped at 4 °C and directly diluted in ice-cold enzyme dilution buffer (1000–10,000-fold) before assay of enzymic activity. In case of radioactive phosphorylation, reactions were carried out in the presence of 50 μM [ $\gamma$ -<sup>32</sup>P]ATP (final activity ~50 μCi/ml) instead of 1 mM ATP and stopped in SDS sample buffer before SDS-PAGE and autoradiography. In order to measure CaM kinase II and/or PKC-mediated <sup>32</sup>P incorporation into astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase, enzyme (0.5 μg) was phosphorylated in the presence of 40 μM [ $\gamma$ -<sup>32</sup>P]ATP (final activity ~500 μCi/ml) for various times (0–10 min) in the presence of CaM kinase II (10 ng), 2 μM CaM, and 10 μM free Ca<sup>2+</sup>, or in the presence of PKC (10 ng), 0.25 mg/ml phosphatidylserine, and 20 μg/ml diacylglycerol. After incubation, each sample was spotted onto P81 phosphocellulose (Whatman), precipitated, and washed in the presence of 75 mM phosphoric acid before counting the radioactivity (31).

## RESULTS

**Modulation of Ins (1,4,5)P<sub>3</sub> 3-Kinase Activity following Receptor Activation in Astrocytes**—Since native Ins (1,4,5)P<sub>3</sub> 3-kinase A activity could be regenerated after SDS-PAGE (see, e.g., Ref. 23), we performed similar experiments with rat astrocytes as starting material. A major 85–90-kDa gel band associated with Ins (1,4,5)P<sub>3</sub> 3-kinase activity from rat astrocytes was detected after SDS-PAGE and regeneration of enzymic activity (Fig. 1A). Similar results were obtained when using enzyme purified from 1321N1 cells or homogenates of rat cortical astrocytes and 1321N1 cells (data not shown). Two distinct antibodies were raised against two peptides chosen in the N- and C-terminal sequence regions of human Ins (1,4,5)P<sub>3</sub> 3-kinase B. Western blot analysis showed a unique 88-kDa band in purified preparation for both sera (Fig. 1B, lanes 1–4). Immunodetected signals were greatly reduced when the sera were used in the presence of the corresponding antigenic peptides (shown for 1321N1 cells) (Fig. 1B, lanes 5 and 6). No signal could be observed with the corresponding preimmune sera (Fig. 1B, lanes 7–10). No recognition could be seen with antibodies raised against isoform A (23) (data not shown). Silver staining of purified astrocytic enzyme from 1321N1 cells revealed a major band with an approximate molecular weight of 88,000 (Fig. 1C).

Carbachol is a well known agonist for muscarinic cholinoreceptor in rat brain cortical astrocytes and in 1321N1 cells, where it mediates an enhancement of PLC activity, the production of Ins (1,4,5)P<sub>3</sub>, Ins (1,3,4,5)P<sub>4</sub>, and intracellular calcium mobilization (7, 8). The same events were observed in cortical astrocytes in response to purinoreceptors P2Y and P2U activation with ATP and UTP (32), but this last response was not observed in 1321N1 cells since this cell line lacks the P2Y receptors (33). Incubation of rat brain cortical astrocytes or 1321N1 cells with carbachol provoked a transient increase in Ins (1,4,5)P<sub>3</sub> 3-kinase activity, i.e. 6–8-fold as compared with basal activity after 15–30 s of incubation with the agonist (Fig. 2). Maximal enzyme activation was achieved at 10 μM carbachol (Table I). Activation with UTP provoked in rat cortical astrocytes the same rapid increase in Ins (1,4,5)P<sub>3</sub> 3-kinase activity (Fig. 2A) with maximal effect at 10 μM agonist (Table I). No effect of UTP was observed in 1321N1 cells (Fig. 2B and Table I). Histamine was much less potent (1.3–1.6-fold stimulation) (Fig. 2). There was no effect of thrombin (data not shown). Preincubation with okadaic acid of cortical astrocytes or 1321N1 cells before receptor activation provided a maximal and more rapid activation of Ins (1,4,5)P<sub>3</sub> 3-kinase (i.e. 10-fold) (Fig. 2). TPA also provoked stimulation of Ins (1,4,5)P<sub>3</sub> 3-kinase activity, i.e. 2–2.5-fold, in cortical astrocytes and 1321N1



**FIG. 1. Regeneration and immunodetection by specific polyclonal anti-(human Ins(1,4,5)P<sub>3</sub> 3-kinase B) antibodies of purified astrocytic Ins(1,4,5)P<sub>3</sub> 3-kinase.** A, Purified rat astrocytic enzyme (0.5 μg, 0.65 nmol/min) was separated by SDS-PAGE before cutting the gel and assaying enzymic activity at 5 μM Ins(1,4,5)P<sub>3</sub>. The major active gel band at 85–90 kDa is indicated by an arrow. B, Enzyme purified from 1321N1 cells (lanes 1, 3, 5–7, and 9) or from rat cortex astrocytes (lanes 2, 4, 8, and 10) were separated by SDS-PAGE, electrophoretically transferred, and immunodetected with antibodies raised against a C-terminal peptide (lanes 1, 2, and 5) or a N-terminal peptide (lanes 3, 4, and 6) derived from the human Ins(1,4,5)P<sub>3</sub> 3-kinase B primary structure. Competition experiments were performed by immunodetecting enzyme from 1321N1 cells in the presence of 5 μg of the corresponding peptides (lanes 5 and 6). Immunodetections were also performed with the corresponding preimmune sera (lanes 7–10). C, Enzyme purified from 1321N1 cells (40 ng, 50 pmol/min) was electrophoretically separated and silver-stained. The 88-kDa band is indicated by an arrow.

cells, whereas forskolin did not provoke any change in enzymic activity (Table I). In both cell systems, activation of Ins (1,4,5)P<sub>3</sub> 3-kinase was related to an increase in  $V_{max}$  with no change in the apparent  $K_m$  value for Ins (1,4,5)P<sub>3</sub> ( $K_m = 1.5 \pm 0.4 \mu M$ ) (data not shown).

**Effect of CaM Kinase II and PKC Inhibitors on Ins (1,4,5)P<sub>3</sub> 3-Kinase Activation**—We investigated the effect of specific membrane-permeable PKC and CaM kinase II inhibitors on

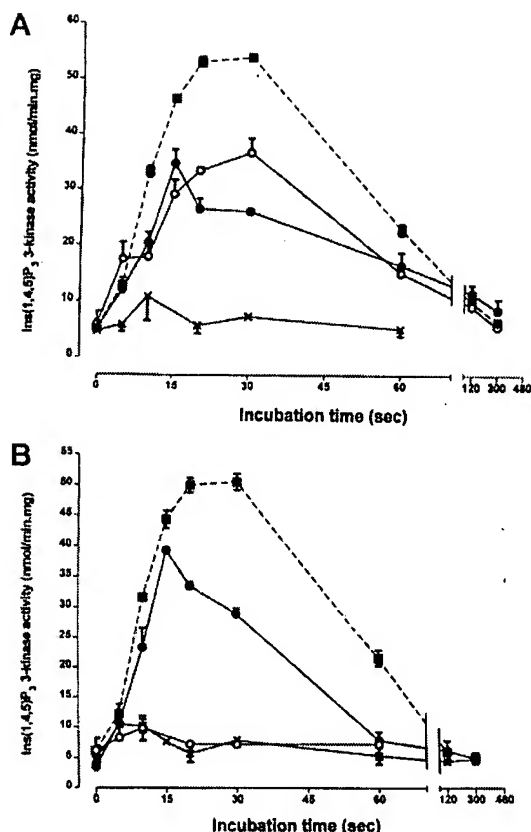


FIG. 2. Effect of  $\text{Ca}^{2+}$ -raising agonists and okadaic acid on  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase activity in rat brain cortical astrocytes (A) and in human 1321N1 astrocytoma cells (B). The two cell types (two dishes per condition) were incubated at  $37^\circ\text{C}$  at the indicated times with  $10\ \mu\text{M}$  carbachol (●), UTP (○), or histamine (×). Okadaic acid ( $75\ \text{nM}$ ) (■) was added for 30 min before agonist stimulation, i.e.  $10\ \mu\text{M}$  carbachol. Astrocytic cells were then lysed in the presence of protease and phosphatase inhibitors, and enzyme activity was measured at  $5\ \mu\text{M}$   $\text{Ins}(1,4,5)\text{P}_3$ . Results are means of triplicates  $\pm$  S.D. of one representative experiment out of four.

the activation of astrocytic  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase in intact cells. Preincubation in the presence of increasing concentrations (up to  $500\ \text{nM}$ ) of calphostin C (potent inhibitor of PKC) before agonist stimulation partially prevented (up to 40%) activation induced by cell stimulation by carbachol (Fig. 3). Additionally, preincubation of both cell types with increasing concentrations (up to  $2\ \mu\text{M}$ ) of two potent CaM kinase II inhibitors, i.e. KN-93 and KN-62, prevented agonist-mediated activation of  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase up to 85% (Fig. 3). The same results were obtained in UTP ( $10\ \mu\text{M}$ )-stimulated rat cortical astrocytes (data not shown). Direct enzyme phosphorylation was evidenced in rat cortical astrocytes prelabeled with  $[^{32}\text{P}]$  orthophosphate and incubated with an agonist (carbachol or UTP) to stimulate  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase activity (Fig. 4).  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase was purified by CaM-Sepharose specifically eluted in the presence of Triton X-100 and EGTA, and analyzed by SDS-PAGE. Enzyme activation coincided with phosphate incorporation into a 88-kDa protein band (Fig. 4). Maximal  $^{32}\text{P}$  incorporation occurred after incubation of astrocytes with  $10\ \mu\text{M}$  carbachol for 30 s or with  $10\ \mu\text{M}$  UTP for 30 s (Fig. 4). Similar results were observed after stimulation of 1321N1 cells by carbachol but UTP did not present any effect (data not shown). Preincubation with okadaic acid ( $75\ \text{nM}$ ) before receptor activation potentiated the phosphate incorporation into the 88-kDa enzyme in both cell systems (Fig. 4).  $^{32}\text{P}$  incorporation was also observed after incubation of both cell types with TPA

TABLE I  
Effect of  $\text{Ca}^{2+}$ -raising agonists, TPA and forskolin on  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase activity in rat brain cortex astrocytes and 1321N1 cells

Enzyme activity was assayed at  $5\ \mu\text{M}$   $\text{Ins}(1,4,5)\text{P}_3$ . 100% enzyme activity corresponds to 4.8 and  $5.2\ \text{nmol/min} \times \text{mg}$  in rat cortex astrocytes and 1321N1 cells, respectively. Each value is the mean of duplicates  $\pm$  S.D. The results are from one representative experiment out of six.

Incubation conditions	$\text{Ins}(1,4,5)\text{P}_3$ 3-kinase activity	
	Rat brain cortex astrocytes	1321N1 cells
No agent	100 $\pm$ 8	100 $\pm$ 5
Carbachol		
0.1 $\mu\text{M}$ (20 s)	163 $\pm$ 10	149 $\pm$ 8
1 $\mu\text{M}$	265 $\pm$ 9	270 $\pm$ 11
5 $\mu\text{M}$	484 $\pm$ 19	459 $\pm$ 10
10 $\mu\text{M}$	823 $\pm$ 36	799 $\pm$ 21
50 $\mu\text{M}$	790 $\pm$ 22	716 $\pm$ 21
UTP		
0.1 $\mu\text{M}$ (20 s)	158 $\pm$ 5	107 $\pm$ 13
1 $\mu\text{M}$	258 $\pm$ 9	105 $\pm$ 15
5 $\mu\text{M}$	459 $\pm$ 16	94 $\pm$ 5
10 $\mu\text{M}$	813 $\pm$ 28	113 $\pm$ 9
50 $\mu\text{M}$	740 $\pm$ 12	99 $\pm$ 12
TPA		
0 s (400 nM)	95 $\pm$ 5	104 $\pm$ 8
30 s	121 $\pm$ 11	91 $\pm$ 8
1 min	206 $\pm$ 1	192 $\pm$ 6
2 min	240 $\pm$ 18	229 $\pm$ 14
5 min	126 $\pm$ 18	109 $\pm$ 20
Forskolin		
30 s (10 $\mu\text{M}$ )	102 $\pm$ 8	97 $\pm$ 3
1 min	93 $\pm$ 13	97 $\pm$ 19
3 min	99 $\pm$ 4	108 $\pm$ 8
5 min	105 $\pm$ 11	102 $\pm$ 4
15 min	100 $\pm$ 3	95 $\pm$ 12

for at least 3 min (Fig. 5). On the other hand, preincubation with KN-93 or KN-62 before receptor activation prevented  $^{32}\text{P}$  incorporation into the enzyme in a dose-dependent manner. Calphostin C also prevented phosphate incorporation (Fig. 5). No enzyme phosphorylation could be seen in response to forskolin (data not shown).

**Distribution of  $\text{Ins}(1,4,5)\text{P}_3$  3-Kinase Activity and Immunodetection after Receptor Activation**—Intracellular distribution of astrocytic  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase activity was studied after *in vivo* phosphorylation and activation of the enzyme in intact astrocytes.  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase activity was assayed in stimulated cortical astrocytes or 1321N1 cells after separation of cytosolic and particulate fractions by centrifugation at  $100,000 \times g$  for 1 h. In quiescent rat cortex astrocytes, enzymic activity was soluble at approximately 65% and particulate at 35% (Fig. 6A). This was also observed in 1321N1 cells (Fig. 6B). Under conditions where the enzyme was activated in response to carbachol or UTP, enzymic activity was mainly measured in the particulate fraction and only a residual activity (approximately 10% of total cell activity) was detected in the soluble fraction after maximal activation at 30 s (Fig. 6). This was not observed in 1321N1 cells in response to UTP (Fig. 6B). Western blot analysis of purified  $\text{Ins}(1,4,5)\text{P}_3$  3-kinase B from 1321N1 cells permitted to observe a relocation of the enzyme to the particulate fraction in response to carbachol (Fig. 7B). In quiescent cells, approximately the same amounts of enzyme were detected in soluble and particulate fractions, whereas the enzyme was relatively more abundant in the particulate fraction after cell stimulation by carbachol ( $10\ \mu\text{M}$ ) for 30 s (Fig. 7B). Relocation occurred when the enzyme was maximally phosphorylated and activated (see Figs. 2 and 4).

**Sensitivity to the  $\text{Ca}^{2+}$ -CaM Complex**—When enzymic activity was determined in the presence of increasing concentrations of CaM ( $0$ – $2\ \mu\text{M}$ ), *in vivo* phosphorylation of  $\text{Ins}(1,4,5)\text{P}_3$

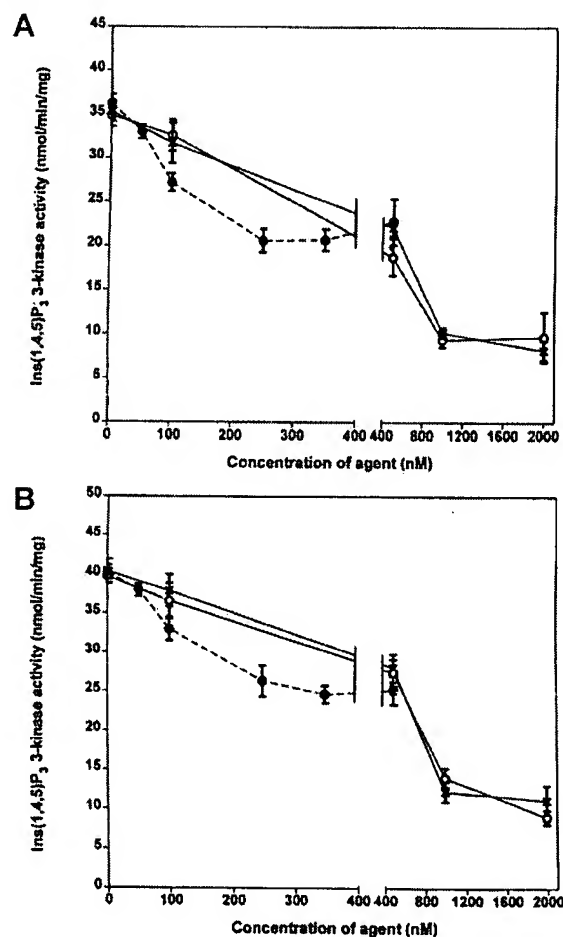


FIG. 3. Effect of PKC inhibitor calphostin C and two CaM kinase II inhibitors KN-93 and KN-62 on Ins(1,4,5)P<sub>3</sub> 3-kinase activation in stimulated rat brain cortical astrocytes (A) and 1321N1 cells (B). Calphostin C (●), KN-62 (○), and KN-93 (×) inhibitors were added for 30 min at the indicated concentrations before stimulation of both cell types with 10  $\mu$ M carbachol for 30 s. Enzyme activity was assayed at 5  $\mu$ M Ins(1,4,5)P<sub>3</sub>. Each value is the mean of duplicates  $\pm$  S.D. The results are from one representative experiment out of five.

3-kinase in rat cortex astrocytes stimulated by carbachol (as well as in stimulated 1321N1 cells; data not shown) did not provoke any change in CaM sensitivity as compared with the enzyme in quiescent cells (Fig. 8). Half-maximal stimulation of astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase activity was reached at approximately 17 nM CaM for non-phosphorylated enzyme as well as for phosphorylated enzyme (Fig. 8). In the first case, the maximal stimulation factor by the Ca<sup>2+</sup>-CaM complex was 14-fold, whereas in the second case, this factor was only 8-fold, indicating that the maximal enzyme activation (due to enzyme phosphorylation and CaM binding) was about 70-fold (Fig. 8).

**In Vitro Phosphorylation of Astrocytic Ins (1,4,5)P<sub>3</sub> 3-Kinase B by CaM Kinase II and PKC**—Since *in vivo* phosphorylation of Ins (1,4,5)P<sub>3</sub> 3-kinase isolated from astrocytes was inhibited by potent CaM kinase II and PKC inhibitors, we investigated *in vitro* phosphorylation of astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase by CaM kinase II and PKC. Basal activity was stimulated 14-fold by the Ca<sup>2+</sup>-CaM complex when added in the activity assay (Fig. 9A, black column). CaM kinase II-catalyzed phosphorylation and direct CaM binding resulted in an increase in Ins (1,4,5)P<sub>3</sub> 3-kinase activity in the presence of 10  $\mu$ M free Ca<sup>2+</sup> and 2  $\mu$ M CaM, *i.e.* 35–40-fold, whereas PKC alone induced an inhibitory effect (50%) in the presence of its cofactors (as com-

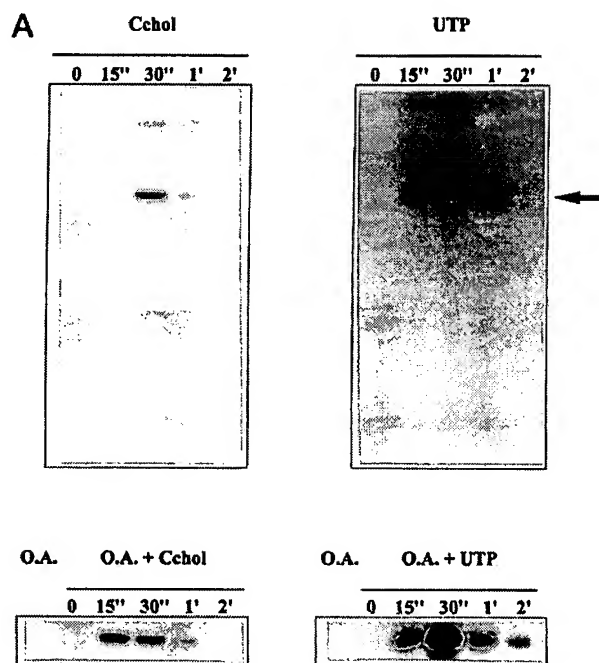


FIG. 4. Okadaic acid-sensitive phosphorylation of Ins(1,4,5)P<sub>3</sub> 3-kinase in rat brain cortical astrocytes. Cells were preincubated with [<sup>32</sup>P]orthophosphate for 2 h before incubation at the indicated times in the presence of various agents, *i.e.* carbachol (Cchol), UTP, or okadaic acid (O.A.). Cells were lysed in the presence of protease and phosphatase inhibitors and enzyme was purified by CaM-Sepharose in each condition. The 88-kDa enzyme was detected by autoradiography following SDS-PAGE. Carbachol and UTP were at 10  $\mu$ M and okadaic acid at 75 nM. Okadaic acid was added for 30 min before Ca<sup>2+</sup>-raising agonist stimulation. Entire autoradiographies are shown in the two first stimulation conditions with an arrow indicating the 88-kDa protein.

pared with basal activity measured after preincubation in the absence of any protein kinase). When the experiment was performed in the presence of CaM kinase II and PKC together and corresponding effectors, a maximal stimulation of Ins (1,4,5)P<sub>3</sub> 3-kinase activity was observed, *i.e.* approximately 60–70-fold as compared with the control (Fig. 9A). This last stimulation effect was not affected when the preincubated enzyme was assayed in the presence of the Ca<sup>2+</sup>-CaM complex (data not shown). We checked that *in vitro* modulation of Ins (1,4,5)P<sub>3</sub> 3-kinase activity by PKC and/or CaM kinase II was associated with <sup>32</sup>P incorporation at the level of an 88-kDa protein after SDS-PAGE and autoradiography (data not shown). Similar results were obtained for *in vitro* phosphorylation of purified Ins (1,4,5)P<sub>3</sub> 3-kinase from 1321N1 cells (data not shown). Moreover, stoichiometry of Ins (1,4,5)P<sub>3</sub> 3-kinase B phosphorylation by CaM kinase II and/or PKC was estimated *in vitro*. CaM kinase II and PKC provoked an incorporation of <sup>32</sup>P to the enzyme with a molar ratio of 1.1 after 2 min and of 2.3 after 5 min, respectively. When used together, 1 mol of enzyme was labeled with 3.3 mol of <sup>32</sup>P after 5 min, indicating that distinct sites were phosphorylated independently by these two protein kinases (Fig. 9B).

#### DISCUSSION

On Western blots, two distinct antibodies raised against two distinct peptides chosen in the primary structure of human Ins (1,4,5)P<sub>3</sub> 3-kinase B recognized the purified 88-kDa Ins (1,4,5)P<sub>3</sub> 3-kinase isoform in rat astrocytes and human astrocytoma cells. From this, we concluded that the isoform present in astrocytic cells is isoform B. Regulation of Ins (1,4,5)P<sub>3</sub> 3-kinase could be driven by two types of mechanisms: direct



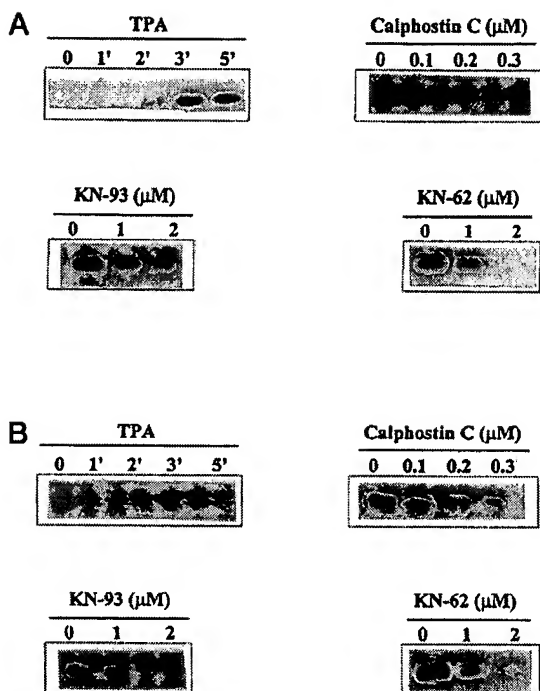


FIG. 5. Effects of TPA, calphostin C, KN-93, and KN-62 on phosphorylation of Ins(1,4,5)P<sub>3</sub> 3-kinase in rat brain cortical astrocytes (A) and in human 1321N1 astrocytoma cells (B). Cells were preincubated with [<sup>32</sup>P]orthophosphate for 2 h before incubation in the presence of the various agents. The procedures for lysis and enzyme purification are the same as described in the legend of Fig. 4. The 88-kDa enzyme was detected by autoradiography. TPA was added at 400 nM for 0–5 min. Calphostin C, KN-93, and KN-62 were added at the indicated concentrations for a 30-min preincubation before cell stimulation with carbachol at 10 μM for 30 s.

binding of the Ca<sup>2+</sup>-CaM complex and enzyme phosphorylation by protein kinases. In response to a Ca<sup>2+</sup>-raising agent, PKC and CaM kinase II provoke an okadaic acid-sensitive phosphorylation and activation of Ins (1,4,5)P<sub>3</sub> 3-kinase in intact astrocytes. The Ca<sup>2+</sup>-CaM complex contributes to maximal stimulation of Ins (1,4,5)P<sub>3</sub> 3-kinase activity. This coincided with a redistribution of the enzyme to the particulate fraction and no change in sensitivity to the Ca<sup>2+</sup>-CaM complex. In the present study, we provided evidence that Ins (1,4,5)P<sub>3</sub> 3-kinase isoform B expressed in astrocytes is activated by both PKC and CaM kinase II. This was shown in primary cultures of rat cortical astrocytes stimulated through the muscarinic and purinergic pathways. Similar results were obtained in 1321N1 cells stimulated through the muscarinic pathway. Phosphorylation of the enzyme in astrocytic cells was potentiated and more rapid in the presence of okadaic acid, as shown for activation and <sup>32</sup>P labeling of the enzyme. This could be explained by the sensitivity of Ins (1,4,5)P<sub>3</sub> 3-kinase B or of the protein kinases mediating its activation to an okadaic-sensitive protein phosphatase. The control by dephosphorylation and deactivation of CaM kinase II by specific okadaic acid-sensitive protein phosphatases has been reported (34, 35). In rat brain, CaM kinase II was an excellent substrate for brain protein phosphatase 2A and the decrease of the CaM kinase II phosphorylation level observed in depolarized hippocampal synaptosomes was blocked in the presence of okadaic acid (35). The presence of Ins (1,4,5)P<sub>3</sub> 3-kinase A in rat brain, especially in neurons, as the major expressed isoform has been previously reported (15, 20). A pre- and post-embedding immunoelectron microscopic study made it possible to show that rat Ins (1,4,5)P<sub>3</sub> 3-kinase A was present at highest levels in the dendritic spines of cerebellar

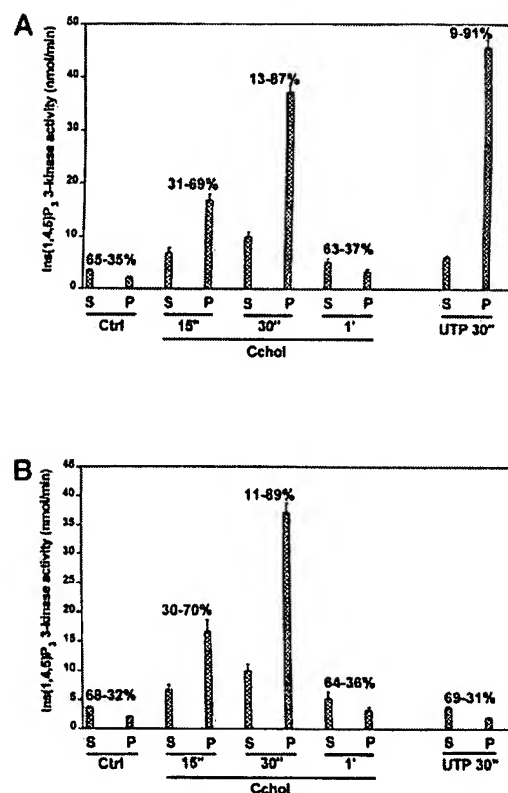
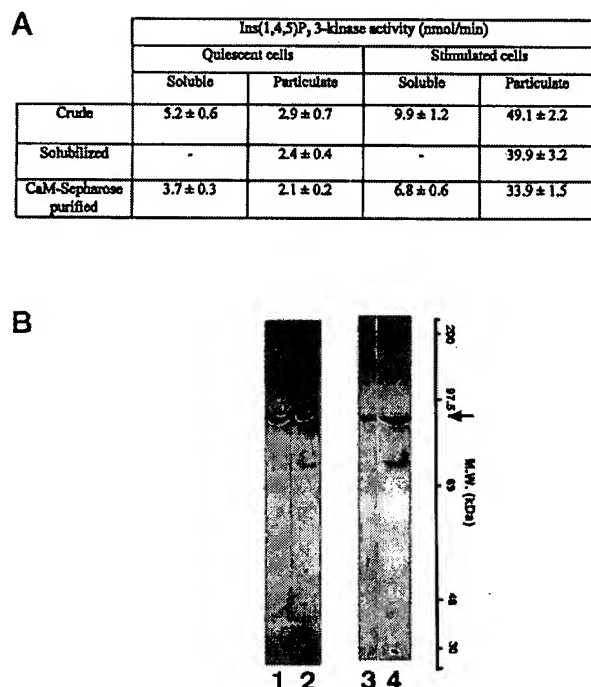


FIG. 6. Subcellular distribution of Ins(1,4,5)P<sub>3</sub> 3-kinase activity before and after enzyme activation in intact astrocytes. Soluble (S) and particulate (P) fractions were separated by centrifuging at 100,000 × *g* for 1 h starting from homogenates prepared from rat cortex astrocytes (A) or from 1321N1 cells (B). Enzyme activity was assayed at 5 μM Ins(1,4,5)P<sub>3</sub>. Homogenates were prepared from quiescent cells (Ctrl) and from the cells stimulated by 10 μM carbachol (Cchol) at the indicated times or by UTP for 30 s.

Purkinje cells and hippocampal CA1 pyramidal cells at the level of the cytoplasmic matrix (36, 37). Expression of messenger RNA corresponding to isoenzyme B has been reported in rat astrocytic cells (21), which is consistent with our data obtained in this study.

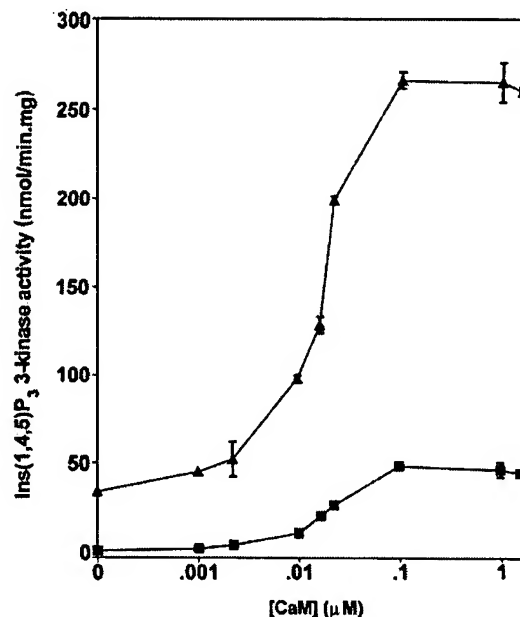
The regulation of Ins (1,4,5)P<sub>3</sub> 3-kinase by phosphorylation is thus quite general, although the present study reveals that the functional consequences of this novel regulation of astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase by CaM kinase II and PKC are distinct from those observed for isoform A in rat brain cortical slices (summarized in Table II): (a) the degree of activation was higher (up to 10-fold in comparison with 5-fold in homogenates, respectively); (b) there was no change in sensitivity to the Ca<sup>2+</sup>-CaM complex, whereas isoform A presented a 25-fold decrease in *K<sub>d</sub>* for this complex; and, finally, (c) isoenzyme B was redistributed to the particulate fraction when phosphorylated and activated (shown in this study), whereas isoform A remained soluble in the same separation conditions in rat brain cortical slices (data not shown). The higher degree of activation could explain the differences in the stimulation of the phosphoinositide cycle by amine neurotransmitters (*e.g.* carbachol) that have been revealed in cultured rat forebrain neurons and astrocytes (8). Measurements of [<sup>3</sup>H]inositol phosphates in the two last systems suggested that the metabolic pathway involving Ins (1,4,5)P<sub>3</sub> 3-kinase prevails in astrocytes and the one involving Ins (1,4,5)P<sub>3</sub> 5-phosphatase prevails in neurons. Recent theoretical work simulated the effects of Ins (1,4,5)P<sub>3</sub> 3-kinase and 5-phosphatase activities on the temporal pattern of Ca<sup>2+</sup> oscillations (38). It was predicted, on the basis of the



**FIG. 7. Relocation of Ins(1,4,5)P<sub>3</sub> 3-kinase before and after enzyme activation in intact astrocytes.** A, homogenates were prepared from 1321N1 quiescent cells and from the cells stimulated by 10  $\mu$ M carbachol for 30 s. Soluble and particulate fractions were separated by centrifuging at 100,000  $\times g$  for 1 h. The particulate fractions were solubilized in 1% Triton X-100 for 1 h at 4  $^{\circ}$ C. The soluble and the solubilized particulate fractions (1 ml) were purified by CaM-Sepharose, and the active fractions eluted in the presence of 2 mM EGTA/1% Triton X-100 were concentrated. Enzyme activity was assayed at 5  $\mu$ M Ins(1,4,5)P<sub>3</sub>. B, concentrated fractions issued from CaM-Sepharose purification of soluble (1, 3) and particulate (2, 4) fractions of quiescent (1, 2) and stimulated (3, 4) 1321N1 cells were separated by SDS-PAGE and immunodetected in the presence of the anti-human Ins(1,4,5)P<sub>3</sub> 3-kinase B antibodies dressed against the C-terminal peptide (dilution 1/750). The band with an apparent molecular weight of 88,000 is indicated by an arrow.

Ins (1,4,5)P<sub>3</sub> 3-kinase stimulation by the Ca<sup>2+</sup>-CaM complex and the experimentally determined kinetic characteristics of kinase isoform A and phosphatase type I, that Ins (1,4,5)P<sub>3</sub> 5-phosphatase primarily controls the Ins (1,4,5)P<sub>3</sub> levels and so the occurrence and frequency of Ca<sup>2+</sup> oscillations. However, this model predicted that high activation (more than 20-fold) of Ins (1,4,5)P<sub>3</sub> 3-kinase, as is the case for the astrocytic enzyme, may provoke a large contribution of Ins (1,4,5)P<sub>3</sub> 3-kinase in the control of Ins (1,4,5)P<sub>3</sub> and Ins (1,3,4,5)P<sub>4</sub> levels as well as in the Ca<sup>2+</sup> oscillations. Concerning its subcellular distribution, previously reported immunodetection analysis have indicated that rat Ins (1,4,5)P<sub>3</sub> 3-kinase B exists as a cytosolic protein and was also associated with peripheral membranes of the cytosolic face of the extended endoplasmic reticulum network in transfected cells (39). Our data obtained in quiescent astrocytes are consistent with a particulate and cytosolic distribution (35% and 65%, respectively) of Ins (1,4,5)P<sub>3</sub> 3-kinase B activity. We showed by activity assay and Western blot analysis that the distribution of this isoform is modified, *i.e.* relocation to the membrane fraction, in response to the stimulation of astrocytic cells by Ca<sup>2+</sup>-raising agents. This could be a mechanism of concentrating the enzyme at specific membranes directly involved in Ca<sup>2+</sup> homeostasis, *e.g.* endoplasmic reticulum membranes.

As shown in the present study, regulation mechanisms of astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase B are more complex than for neuronal Ins (1,4,5)P<sub>3</sub> 3-kinase A, since it requires at least a



**FIG. 8. CaM dependence of astrocytic Ins(1,4,5)P<sub>3</sub> 3-kinase activity before and after phosphorylation in intact cells.** Rat cortical astrocytes were incubated in the presence (▲) or absence (■) of 10  $\mu$ M carbachol for 30 s at 37  $^{\circ}$ C. Enzymic activity of homogenates was assayed at 5  $\mu$ M Ins(1,4,5)P<sub>3</sub> and 10  $\mu$ M free Ca<sup>2+</sup> with increasing concentrations of CaM (0–2  $\mu$ M). Results are means of triplicates  $\pm$  S.D.

dual phosphorylation by two distinct protein kinases, *i.e.* CaM kinase II and PKC, and so phosphorylation at multiple sites. CaM kinase II and PKC are multifunctional enzymes catalyzing phosphorylation of many proteins, presenting a wide tissue distribution and being particularly abundant in brain, *e.g.* in astrocytic cells (40–42). CaM kinase II is a major mediator of dynamically organized Ca<sup>2+</sup> oscillations and actions. Intracellular distributions of the signaling by CaM kinase II and Ca<sup>2+</sup> were studied in the same astrocytes (43). These results indicated that local spatio-temporal Ca<sup>2+</sup> signals induced protein phosphorylation (*e.g.* vimentin) by CaM kinase II in the same cellular compartment in astrocytes and that Ca<sup>2+</sup> waves induced global protein phosphorylation by CaM kinase II. The abundance of total PKC isoforms has been reported in rat astrocytes and phorbol esters cause a rapid translocation of PKC activity from cytosol to membrane compartments in this cell system (44, 45). Interestingly, Ras-GAP1<sup>IP4BP</sup> presents a specific mRNA distribution in rat brain, since it is highly expressed in the hippocampus and cerebellum, specially in neurons and oligodendrocytes, but not in astrocytes (46). This is compatible with cell type-specific roles of the second messenger Ins (1,3,4,5)P<sub>4</sub>, for which our data suggest its levels are controlled by distinct isoenzymes of Ins (1,4,5)P<sub>3</sub> 3-kinase presenting a specific cellular and subcellular distribution, as well as specific regulation mechanisms by phosphorylation.

Ins (1,4,5)P<sub>3</sub> 3-kinase A was phosphorylated *in vitro* and *in vivo* by CaM kinase II at the level of one unique residue, *i.e.* Thr-311 (in the primary structure of the human isoenzyme). This residue is one of the two predicted phosphorylation consensus sites for CaM kinase II previously identified in the sequence of Ins (1,4,5)P<sub>3</sub> 3-kinase A (*i.e.* Arg-Ala-Val-Thr<sup>311</sup> for consensus site Arg/Lys-X-X-Ser/Thr; Refs. 15 and 47) (27). Interestingly, this residue is conserved in the primary structure of human and rat Ins (1,4,5)P<sub>3</sub> 3-kinase B (17–19), suggesting the novel phosphorylation mechanism presented here to involve at least this common residue as target for CaM kinase II-mediated phosphorylation and enzyme activation. Many

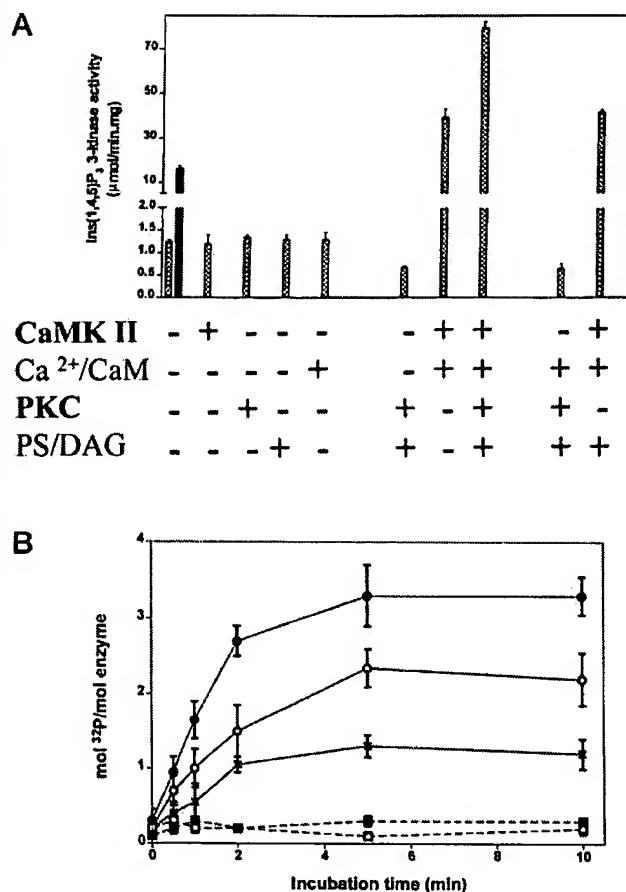


FIG. 9. Effect on enzymic activity and stoichiometry of phosphate incorporation related to *in vitro* phosphorylation of astrocytic Ins(1,4,5)P<sub>3</sub> 3-kinase by CaM kinase II and PKC. A, purified rat astrocytic Ins(1,4,5)P<sub>3</sub> 3-kinase (100 ng) was preincubated during 5 min at 37 °C and pH 7.4 in the presence or absence of 2 ng of protein kinases (CaM kinase II or PKC). Free Ca<sup>2+</sup> and CaM were at 10 and 2 μM, respectively. Phosphatidylserine and 1,2-diacylglycerol were at 0.25 mg/ml and 20 μg/ml, respectively. After incubation, samples were diluted 1000–10,000-fold before assay of enzymic activity at 5 μM Ins(1,4,5)P<sub>3</sub>. In the case of preincubation in the absence of any agent, enzymic activity was also assayed in the presence of 10 μM Ca<sup>2+</sup> and 0.1 μM CaM (black column). PS, DAG, and CaMKII are for phosphatidylserine, 1,2-diacylglycerol, and CaM kinase II, respectively. Results are means of triplicates ± S.D. B, CaM kinase II and/or PKC-catalyzed <sup>32</sup>P incorporation into astrocytic Ins(1,4,5)P<sub>3</sub> 3-kinase has been measured by phosphorylating enzyme (0.5 μg) with CaM kinase II (10 ng) (×) or PKC (10 ng) (○) and 40 μM [<sup>32</sup>P]ATP (final activity ~500 μCi/ml) for various times (0–10 min) in the presence of 2 μM CaM and 10 μM free Ca<sup>2+</sup> or 0.20 mg/ml phosphatidylserine and 20 μg/ml 1,2-diacylglycerol, respectively. The phosphorylation reaction was also performed with both protein kinases and related cofactors together (●). The autophosphorylation state of CaM kinase II (■) and PKC (□) in the presence of their cofactors was estimated in the absence of 3-kinase. After each incubation time, the enzyme was precipitated onto P81 phosphocellulose with 75 mM phosphoric acid before counting radioactivity. Results are means of triplicates ± S.D.

phosphorylation consensus sites for PKC are found in the primary structures of rat and human Ins (1,4,5)P<sub>3</sub> 3-kinase B: 10 sites in the human sequence (*i.e.* Ser-8, Ser-71, Ser-184, Ser-216, Ser-278, Ser-332, Ser-391, Ser-407, Ser-423, and Ser-508) and 8 sites in the rat sequence (*i.e.* Ser-8, Ser-172, Ser-315, Ser-374, Ser-390, Ser-406, Ser-491, and Thr-261). Six of them are conserved between both species (in *italic*) and only one is conserved in human sequence of isoform A (in **bold**). We showed here that Ins (1,4,5)P<sub>3</sub> 3-kinase B is not only a target of CaM kinase II, it is also a substrate of PKC to observe maximal enzyme activation in intact astrocytic cells. Recombinant rat

TABLE II  
Regulatory properties of Ins(1,4,5)P<sub>3</sub> 3-kinase isoforms A and B

Neuronal Isoform A	Astrocytic Isoform B
Mass = 53 kDa (Western blot and activity regeneration)	Mass = 88 kDa (Western blot and activity regeneration)
Low sensitivity to CaM (2-fold activation)	High sensitivity to CaM (15-fold activation)
<i>In vitro</i> activation by CaM binding and phosphorylation by CaM kinase II: 10-fold	<i>In vitro</i> activation by CaM binding and phosphorylation by PKC and CaM kinase II: >60-fold
No effect of the PKA in intact cells	No effect of the PKA in intact cells
<i>In vivo</i> activation in rat brain cortical slices by phosphorylation: 5-fold	<i>In vivo</i> activation in intact astrocytes by phosphorylation: 10-fold
<i>In vivo</i> phosphorylation by CaM kinase II on a unique threonyl residue	<i>In vivo</i> phosphorylation by PKC and CaM kinase II
Increase in affinity for CaM (25-fold) after phosphorylation	No change in affinity for CaM after phosphorylation
No redistribution of enzyme activity (cytosol)	Enzyme redistribution to the membrane fraction

Ins (1,4,5)P<sub>3</sub> 3-kinase B has been shown to be a substrate of PKC and PKA *in vitro*; both protein kinases provoked a decrease in enzymic activity in the presence of the Ca<sup>2+</sup>-CaM complex (26). Our data in intact astrocytes with TPA and calphostin C revealed that the PKC pathway did not provoke any enzyme inhibition. Instead, maximal phosphorylation and activation of Ins (1,4,5)P<sub>3</sub> 3-kinase B was observed in the presence of PKC and CaM kinase II. Our results indicated also that the effect of PKC may require prior phosphorylation by CaM kinase II to mediate maximal activation of Ins (1,4,5)P<sub>3</sub> 3-kinase B in astrocytes. This is supported by the inhibitory effect of KN-62 and KN-93 inhibitors on the TPA-induced phosphorylation and activation of astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase, as well as the *in vitro* PKC-induced activation of the enzyme, which is observed only in the presence of CaM kinase II. *In vitro* experiments indicated that CaM kinase II and PKC provoke the phosphorylation of one and two phosphorylation sites on astrocytic Ins (1,4,5)P<sub>3</sub> 3-kinase, respectively. Concerning PKC phosphorylation of Ins (1,4,5)P<sub>3</sub> 3-kinase B, this result is in agreement with previously reported *in vitro* experiments where at least two sites were labeled (26). Forskolin did not induce any change in Ins (1,4,5)P<sub>3</sub> 3-kinase activity or <sup>32</sup>P incorporation in cortical astrocytes or 1321N1 cells, indicating that PKA does not seem to be a physiological effector of isoenzyme B.

As for Ins (1,4,5)P<sub>3</sub> 3-kinase isoenzymes in this study, specific cell distribution has also been demonstrated for Ins (1,4,5)P<sub>3</sub> receptor isoforms in brain. Immunohistochemical and biochemical studies in rat central nervous system indicated that Ins (1,4,5)P<sub>3</sub>-induced Ca<sup>2+</sup> release is mediated by receptor isoform type 1 in neuronal cells, whereas it is mainly detected by receptor isoforms 2 and/or 3 in astrocytes (48, 49). Molecular heterogeneity generating specific regulatory mechanisms and subcellular distributions of PLC, Ins (1,4,5)P<sub>3</sub> receptor and Ins (1,4,5)P<sub>3</sub> 3-kinase isoforms may be considered as critical parameters involved in the complex mechanisms that underlie Ca<sup>2+</sup> wave formation and propagation in brain cells, *e.g.* astrocytes. Since no regulatory mechanism involving Ca<sup>2+</sup> has been demonstrated for Ins (1,4,5)P<sub>3</sub> 5-phosphatase type I, the metabolic pathway through highly regulated Ins (1,4,5)P<sub>3</sub> 3-kinase may be the critical route for Ca<sup>2+</sup>-controlled Ins (1,4,5)P<sub>3</sub> and Ins (1,3,4,5)P<sub>4</sub> levels and actions.

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## Additions and Corrections

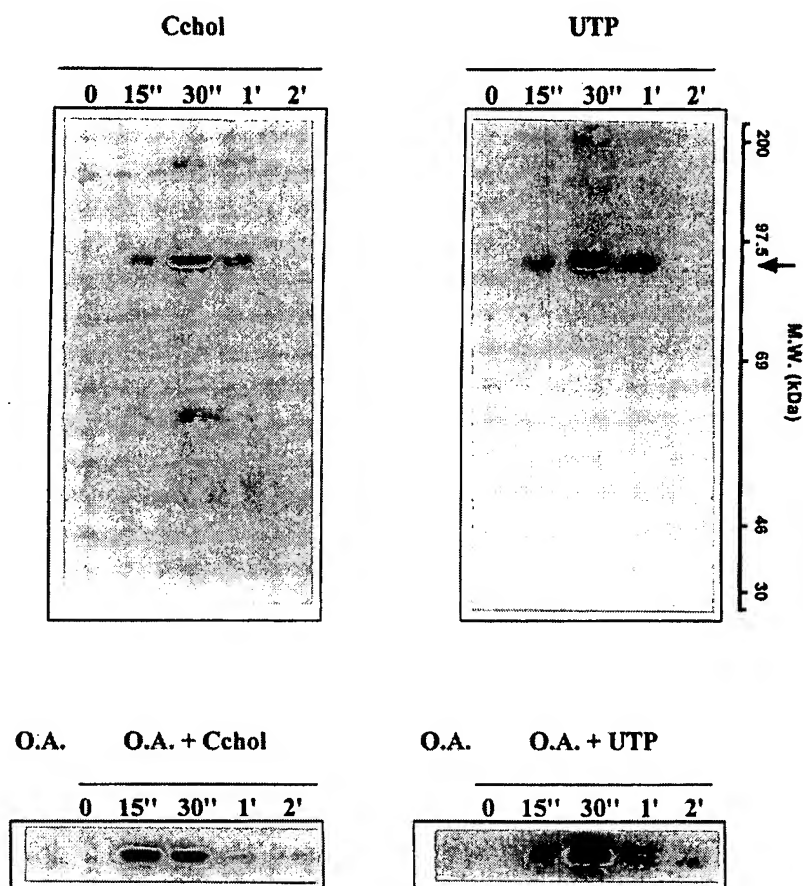
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**Calcium-calmodulin-dependent protein kinase II and protein kinase C-mediated phosphorylation and activation of *D-myo*-inositol 1,4,5-trisphosphate 3-kinase B in astrocytes.**

*David Communi, Valérie Dewaste, and Christophe Erneux*

**Page 14738, Fig. 4:** The molecular mass scale was inadvertently omitted from this figure. The correct figure is shown below.

FIG. 4



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